BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

STIC-ILL

From: Kerr, Janet
Sent: Wednesday, May 09, 2001 2:32 PM
To: STIC-ILL
Subject: references for 09/390,634

Please order the following references:

Burdon et al.
Cells Tissues Organs
Vol. 165, No. 3-4
Pages 131-143
1999

Gottlieb et al.
Cells Tissues Organs
Vol. 165, No. 3-4
Pages 165-172
1999

Rohwedel et al.
Cells Tissues Organs
Vol. 165, No. 3-4
Pages 190-202
1999

Boheler et al.
Cells Tissues Organs
Vol. 165, No. 3-4
Pages 237-245
1999

Thomson et al.
Trends Biotechnol.
Vol. 18, No. 2
Pages 53-57
2000

Nichols et al.
Experimental Cell Research
Vol. 215, No. 1
Pages 237-239
1994

Wiles et al.
Experimental Cell Research
Vol. 247, No. 1
Pages 241-248
1999

Nichols et al.
Experimental Cell Research
Vol. 215, No. 1
Pages 237-239
1994

Wiles et al.
Leukemia
Vol. 11, Suppl. 3
Pages 454-456
1997

Booth et al.
Theriogenology
Vol. 47, No. 1
pp. 240
1997

Thanks,
Janet M. Kerr
305-4055
A.U. 1633
CM1-12A03

Human embryonic stem cell and embryonic germ cell lines

James A. Thomson and Jon S. Odorico

Undifferentiated human embryonic stem (ES) cells and embryonic germ (EG) cells can be cultured indefinitely and yet maintain the potential to form many or all of the differentiated cells in the body. Human ES and EG cells provide an exciting new model for understanding the differentiation and function of human tissue, offer new strategies for drug discovery and testing, and promise new therapies based on the transplantation of ES and EG cell-derived tissues.

The essential characteristics of all stem cells are prolonged self-renewal and the long-term potential to form one or more differentiated cell types. Adult tissues with a high cellular turnover rate, such as the skin, intestine and blood are each sustained by a highly regulated process of stem cell self-renewal, differentiation and death. Stem cells present in adult tissues form only a limited number of cell types, and some tissues completely lack stem cells. However, in the early mammalian embryo there are some cells that have the potential to form any cell type.

Each cell of the cleavage-stage mammalian embryo has the developmental potential to contribute to any embryonic or extra-embryonic cell lineage (Fig. 1). At the blastocyst stage, the embryo forms an inner cell mass (ICM), which can still form any cell of the body, and an outer trophectoderm, which is committed to form part of the placenta[1]. Soon after this, the ICM splits into a primitive endoderm layer that gives rise to extra-embryonic endoderm, and an epiblast layer that gives rise to the embryo proper and to some extra-embryonic derivatives[2]. After implantation and gastrulation, cells become progressively restricted to specific lineages. During this time, a subset of epiblast-derived cells form primordial germ cells (PGCs), which undergo a complex migration to the genital ridges where they become either sperm cells or oocytes[3].

In certain strains of mice and in humans, germ cells occasionally give rise to tumors, termed teratocarcinomas, that contain derivatives of all three embryonic germ layers[4,5]. The undifferentiated stem cell components of teratocarcinomas are termed embryonal carcinoma (EC) cells. Years before the isolation of human EG or ES cells, EC cell lines from both mouse and (later) human teratocarcinomas provided an important *in vitro* model of differentiation. Although the cells of the ICM contribute to all adult tissues, these embryonic cells proliferate and replace themselves in the intact embryo for only a limited period of time before they become committed to specific lineages. Thus, in the unmanipulated embryo, cells of the ICM function as precursor cells, but not as stem cells. However, if ICM cells are removed from their normal embryonic environment and cultured under appropriate conditions, they can proliferate and replace themselves indefinitely, and yet maintain the developmental potential to form advanced derivatives of all three embryonic germ layers, thus satisfying the criteria for stem cells.

Pluripotent mouse ICM-derived cell lines are termed embryonic stem (ES) cells to distinguish their origin from EC cells[6,7]. Similar pluripotent cell lines have been derived from the mouse primordial germ cells of later-stage embryos and, again, to distinguish their origin, primordial germ cell-derived cell lines are now referred to as embryonic germ (EG) cell lines[8,9]. Both mouse ES and EG cells contribute to multiple lineages when formed into chimeras with intact embryos, and will sometimes contribute to the germ line, thereby providing a means of introducing specific genetic alterations into the mouse germ line[10,11].

Given the properties of mouse ES and EG cells, the essential criteria required for applying the term 'ES cell' or 'EG cell' line to human cell lines should include: (1) derivation from the pre- or peri-implantation embryo (ES cells) or primordial germ cells (EG cells); (2) prolonged undifferentiated proliferation (self-renewal); and (3) stable developmental potential following prolonged culture to form advanced derivatives of all three embryonic germ layers. The human cell lines recently derived from blastocysts[12] and from fetal primordial germ cells[13] fulfill these criteria.

Comparison of human EC, ES and EG cells

Human ES, human EC and non-human primate ES cells share a similar morphology that is distinct from human EG cells[12–15]. Human ES (Fig. 2) cells form relatively flat, compact colonies that easily dissociate into single cells in trypsin or in Ca^{2+}- and Mg^{2+}-free medium, whereas human EG cells form tight, more spherical colonies that are refractory to routine dissociation methods, but which more closely resemble the morphology of mouse ES and EG cell colonies. The surface antigens that characterize human EC and ES cells overlap with those expressed by human EG cells. Undifferentiated primate ES cells, human EC cells and human ES cells express stage-specific embryonic antigens 3 and 4 (SSEA-3 and SSEA-4), high molecular weight glycoproteins TRA-1-60 and TRA-1-81, and alkaline phosphatase[12,15–19]. Human EG cells express these markers, but they also express the lactoseries

J.A. Thomson (thomson@primate.wisc.edu) and J.S. Odorico (jon@tx.surgery.wisc.edu) are at the Wisconsin Regional Primate Research Center, and Department of Surgery, School of Medicine (respectively), University of Wisconsin, 1223 Capitol Court, Madison, WI 53715-1299, USA.

0167-7799/00/$ – see front matter PII: S0167-7799(99)01410-9

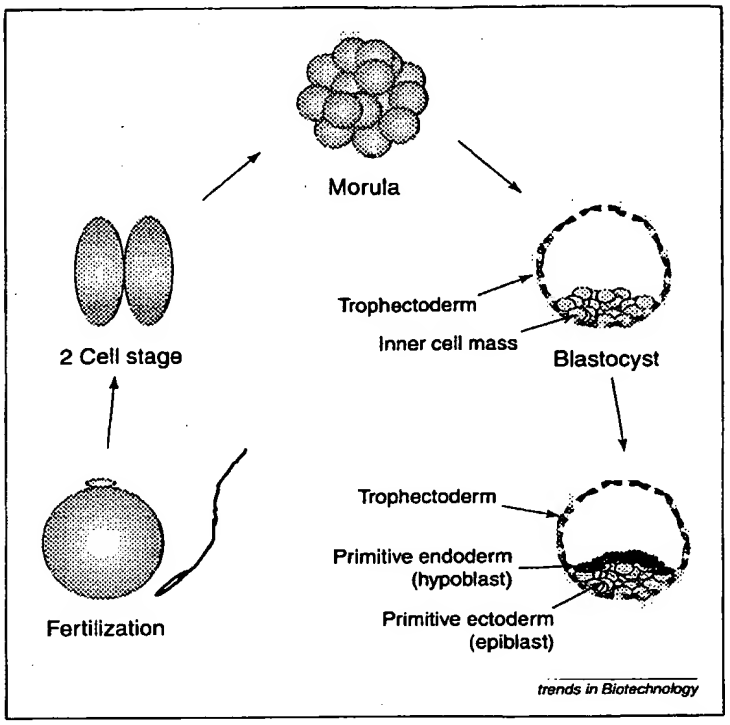

Figure 1

Pre-implantation mammalian development. At the blastocyst stage, the embryo is composed of the inner cell mass and the trophectoderm. The trophectoderm is committed to forming the outer layer of the placenta, but the inner cell mass retains the ability to form any cell of the body. Embryonic stem cells are derived from the isolated inner cell mass; embryonic germ cells are derived from the primordial germ cells (PGCs) of later-stage embryos, either during the migration of the PGCs, or after the PGCs have arrived at the genital ridge.

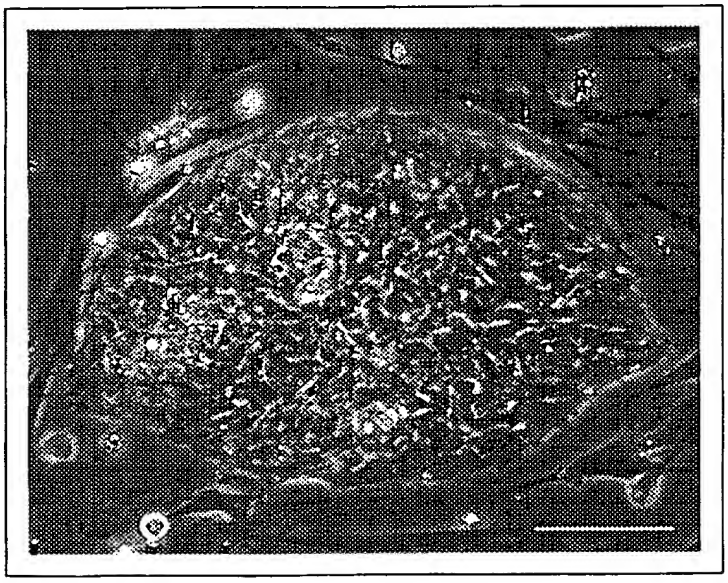

Figure 2

Human embryonic stem (ES) cell colony on mouse fibroblasts. Compared with mouse ES cells and embryonic germ (EG) cells, human and primate ES cells exhibit a flatter morphology with more distinct borders between individual cells. Human EG cells form more-compact colonies, which more closely resemble mouse ES and EG cells in morphology, but are difficult to dissociate into single cells (bar = 50 μm).

glycolipid SSEA-1 (Ref. 13), which is not expressed by human EC and ES cells.

It is not yet clear whether the apparent morphological and phenotypical differences between human ES and EG cells reflect basic biological differences resulting from their different origins, or merely reflect the different culture conditions used to isolate and propagate these two cell types. Human ES cells were isolated and grown on mouse fibroblasts in the presence of serum but in the absence of other exogenous peptide growth factors[12]. Human EG cells were isolated on mouse fibroblast feeder layers in medium that included serum, basic fibroblast growth factor, leukemia inhibitory factor (LIF) and forskolin[13].

The critical factors produced by the fibroblast feeder layers that prevent the differentiation of human ES and EG cells are unknown. Mouse ES cells remain undifferentiated and proliferate in the absence of fibroblasts when grown in the presence of LIF, ciliary neurotropic factor (CNF) or oncostatin M (Refs 20–22). Human ES cells cultured in the presence of LIF and in the absence of fibroblasts uniformly differentiate or die within 1–2 weeks[12]. Some human EC cell lines are feeder-dependent, and LIF, oncostatin M and CNF fail to prevent their differentiation[5]. Given the apparent importance of LIF in sustaining human EG cell proliferation, further work is needed to clarify what role, if any, LIF signaling plays in mediating the undifferentiated proliferation of human ES cells, or whether entirely different signaling pathways are involved.

Similar to their mouse counterparts, human ES cell lines have both more advanced and more consistent developmental potential compared with human EC cell lines. The developmental potential of the latter varies but, in general, is rather limited, possibly reflecting their malignant origin and the presence of severe karyotypic abnormalities[5]. For example, the human EC cell line NTERA2 cl.D1 injected into immunocompromised mice forms teratocarcinomas containing simple tubular structures resembling primitive gut, neural rosettes and tissue resembling neuropile[23]. By contrast, human ES cells injected into immunocompromised mice form benign teratomas with advanced differentiated derivatives representing all three embryonic germ layers[12] (Fig. 3).

Easily identifiable differentiated cells in human ES cell teratomas include smooth muscle, striated muscle, bone, cartilage, gut and respiratory epithelium, keratinizing squamous epithelium, hair, neural epithelium and ganglia. Teratomas formed from human EG cells have not yet been reported, but EG cells allowed to differentiate in culture express cell-surface antigens characteristic of derivatives of the three embryonic germ layers[13]. Unlike human EC cell lines, which are highly aneuploid, both human ES and EG cell lines can maintain a normal karyotype through prolonged periods of culture. Human ES cells express high levels of telomerase[12], an enzyme associated with immortal cell lines, and are capable of undifferentiated proliferation for more than a year, with no observed replicative senescence.

Although ES and EG cell lines both demonstrate a remarkable developmental potential, there appear to be differences between mouse ES and EG cell lines as a result of genomic imprinting. In mammals, genetic contributions from both parents are essential for complete

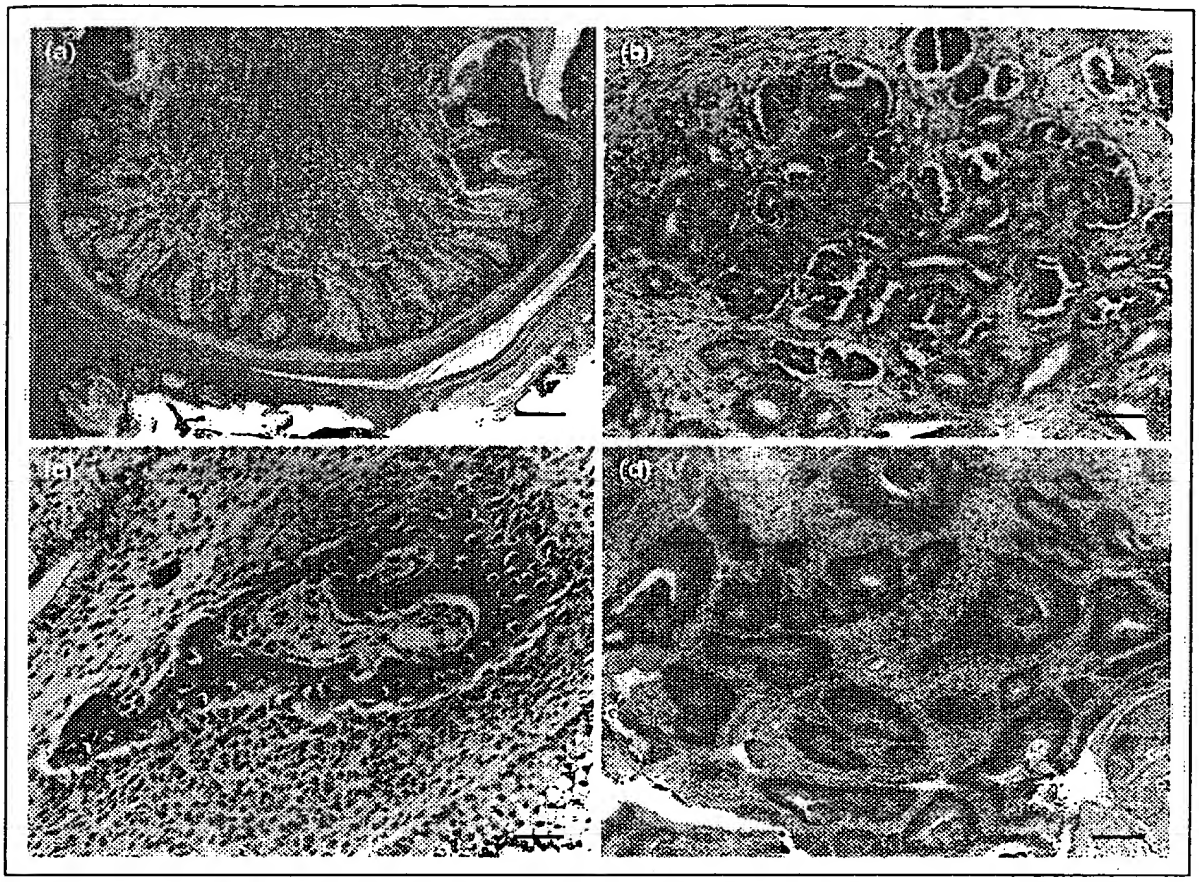

Figure 3

Human embryonic stem cell teratomas. Human embryonic stem cells injected into immunocompromised mice form benign teratomas with abundant differentiation of advanced derivatives of all three embryonic germ layers. **(a)** Gut (bar = 250 μm), **(b)** fetal glomeruli and renal tubules (bar = 67 μm), **(c)** bone (bar = 50 μm), and **(d)** neural ectoderm (bar = 167 μm).

and normal development. This stems from the fact that a limited number of critical genes are differentially expressed depending on whether they are inherited through the male germ line or through the female germ line. Thus, mammalian parthenogenomes, which only have chromosomes from the female germ line, fail to develop to term.

A few select mouse ES cell lines are capable of forming an entire viable fetus[24]. This is accomplished by forming a chimera between ES cells and a tetraploid embryo. In these chimeras, the tetraploid component gives rise to extra-embryonic structures, thus rescuing the ES cells that appear to have a limited ability to form part of the placenta. At least in these select mouse ES cell lines, the normal somatic status of imprinted genes must be faithfully propagated in culture, or complete fetal development would not be possible. However, the status of imprinted genes is altered in some mouse ES cell lines, possibly as a consequence of extended culture, and these ES cells demonstrate abnormal development in chimeras[25]. The somatic imprint of EG cells already appears to be altered at the time of isolation from the primordial germ cells, and EG cells in chimeras demonstrate developmental abnormalities[26,27]. The status of imprinted genes in human ES and EG cells has not yet been investigated. Genomic imprinting could become a critical issue as transplantation therapies based on human ES or EG cells are developed; changes in the status of imprinted genes result in abnormal development, and a relaxation of imprinting of specific genes has been associated with several human malignancies[28–31].

Implications for basic research and human medicine

Human ES cell lines provide a powerful new model for understanding normal human development and therefore can aid in understanding abnormal development. Experimental manipulation of the post-implantation human embryo is ethically unacceptable because of the potential risk to the resulting child, and there can be no functional studies on human post-implantation embryos. Consequently, what is known about human development in the early post-implantation period is based on static histological sections of a few human embryos and on analogy to experimental embryological studies of the mouse. The mouse is the primary model used in mammalian experimental embryology as a result of historical use, well-defined genetics and favorable reproductive characteristics. However, early mouse and human development differ significantly: human and mouse embryos differ in the timing of

embryonic genome expression[32], in the formation, structure and function of the fetal membranes and placenta[33,34], and in the formation of an embryonic disc instead of an egg cylinder[35]. The earliest events of human development are critically involved in human infertility, pregnancy loss and birth defects. Human ES and EG cells offer a new window for the understanding of both these early human developmental events and the pathogenesis of developmental failures.

Human ES and EG cells could provide a potentially unlimited source of differentiated, euploid, non-transformed cells for investigators interested in the normal function and pathology of specific differentiated human cells. Although malignantly transformed human cell lines are commonly available for research, normal primary human cells are less readily available, partly because cells from the adult have a finite replicative lifespan and because some important primary human cell types have proven difficult to culture.

The sustained culture of many human tissue-restricted stem cells has proven particularly elusive. For example, although human bone marrow transplants are routine, continued expansion of pure human hematopoietic stem cells *in vitro* (so that multiple patients can be treated by the same donor bone marrow) is not yet possible, despite years of work towards this goal. Human ES and EG cells offer starting material to study mechanisms of tissue-specific differentiation and regeneration, and may offer the necessary insights that will ultimately allow the sustainable culture of tissue-restricted stem cells. For tissues such as the heart, which completely lack a tissue-specific stem cell, human ES and EG cells will prove even more valuable.

As the Human Genome Project nears completion, identifying the function of novel genes will become increasingly important. Because human ES and EG cells are capable of indefinite proliferation and should be amenable to detailed genetic manipulation, they provide a system for testing the function of human genes during differentiation and in terminally differentiated cells. Although the ability to direct the differentiation of ES and EG cells towards specific lineages is currently limited, considerable progress in mouse ES cell differentiation to neural, hematopoietic and cardiac tissue has been made[36–39]. An on-demand, unlimited supply of human neurons, blood cells, cardiac myocytes or other cells would prove invaluable for drug discovery and toxicity testing.

Human ES and EG cells also offer the promise of new transplantation therapies. When disease results from the destruction or dysfunction of a limited number of cell types, such as in Parkinson's disease (dopaminergic neurons) or juvenile onset diabetes mellitus (pancreatic islet cells), the replacement of those specific cell types by ES cell-derivatives could potentially offer lifelong treatment. However, several obstacles need to be overcome. It will be necessary to direct ES cells more efficiently to specific lineages. For example, although specific culture conditions that promote neural, hematopoietic and cardiomyocyte differentiation of mouse ES cells have been defined to some degree, the resulting population of differentiated cells is always a mixture of different cell types.

Purifying the cells of interest from the mixed population is essential. One strategy is to use a tissue-specific promoter to drive a selectable marker[38]. A potential problem is that transplanted cells have to be integrated into the host tissue in a functionally useful form; replacing infarcted heart muscle or scar tissue with ES cell-derived cardiomyocytes will require that new muscle cells integrate with the existing muscle, contract in a coordinated and mechanically useful manner and develop a new blood supply. Although complex structural integration would be essential for some cell transplants (e.g. neurons and cardiomyocytes), normal functioning of other ES cell-derived transplants will be more independent of such complex tissue interactions (e.g. islet cells).

Another problem is the prevention of immune rejection. Strategies to reduce immune rejection include: establishing 'banks' of major histocompatibility complex antigen-typed human ES or EG cells; genetically altering ES cells to suppress actively the immune response; and using nuclear-transfer techniques, similar to those that created Dolly[40], to produce ES cell lines that are genetically matched for all nuclear genes of a specific nuclear donor. Because malignant transformation of transplanted cells is a concern, testing the safety and efficacy of new transplantation therapies in an accurate model such as rhesus ES cells and rhesus monkeys will be essential[15]. Indeed, for two of the examples mentioned, Parkinson's disease and diabetes mellitus, accurate models are already available in the rhesus monkey[40,41].

Conclusions

Human ES and EG cells offer new models that complement and extend other developmental models for understanding the basic processes that control human development. Mammalian developmental biology is advancing rapidly, and genes that regulate specific developmental events are quickly being identified. Given the pace of these advances, the ability to direct human ES and EG cells to every specific cell type is probably not far off.

The exciting potential of human ES and EG cells is that these advances in basic developmental biology could directly lead to lifelong treatments of serious human diseases. Thus, because of human ES and EG cells, human developmental biology (once largely relegated to static histological descriptions) is in the process of being transformed into a dynamic, experimental discipline with strong relevance to many clinical specialties.

References

1 Winkel, G.K. and Pedersen, R.A. (1988) Fate of the inner cell mass in mouse embryos as studied by microinjection of lineage tracers. *Dev. Biol.* 127, 143–156

2 Gardner, R.L. (1982) Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. Exp. Morphol.* 68, 175–198

3 Lawson, K.A. and Hage, W.J. (1994) Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Found. Symp.* 182, 68–84

4 Damjanov, I. and Solter, D. (1976) Animal model of human disease: teratoma and teratocarcinoma. *Am. J. Pathol.* 83, 241–244

5 Roach, S. *et al.* (1993) Cultured cell lines from human teratomas: windows into tumour growth and differentiation and early human development. *Eur. Urol.* 23, 82–88

6 Evans, M. and Kaufman, M. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156

7 Martin, G. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 78, 7634–7638

8 Matsui, Y. *et al.* (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 70, 841–847

9 Resnick, J.L. *et al.* (1992) Long-term proliferation of mouse primordial germ cells in culture. *Nature* 359, 550–551

10 Bradley, A. *et al.* (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 309, 255–256

11 Stewart, C.L. *et al.* (1994) Stem cells from primordial germ cells can reenter the germ line. *Dev. Biol.* 161, 626–628

12 Thomson, J.A. *et al.* (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147

13 Shamblott, M.J. *et al.* (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. U. S. A.* 95, 13726–13731

14 Thomson, J.A. *et al.* (1995) Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U. S. A.* 92, 7844–7848

15 Thomson, J.A. and Marshall, V.S. (1998) Primate embryonic stem cells. *Curr. Top. Dev. Biol.* 38, 133–165

16 Andrews, P.W. *et al.* (1984) Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma* 3, 347–361

17 Kannagi, R. *et al.* (1983) Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J.* 2, 2355–2361

18 Fox, N.W. *et al.* (1984) Stage-specific embryonic antigen 3 as a marker of visceral extraembryonic endoderm. *Dev. Biol.* 103, 263–266

19 Ozawa, M. *et al.* (1985) SSEA-1, a stage-specific embryonic antigen of the mouse, is carried by the glycoprotein-bound large carbohydrate in embryonal carcinoma cells. *Cell Differ.* 16, 169–173

20 Conover, J.C. *et al.* (1993) Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* 119, 559–565

21 Rose, T.M. *et al.* (1994) Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells *in vitro*. *Cytokine* 6, 48–54

22 Williams, R. *et al.* (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336, 684–687

23 Andrews, P.W. *et al.* (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. *Lab. Invest.* 50, 147–162

24 Nagy, A. *et al.* (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 90, 8424–8428

25 Dean, W. *et al.* (1998) Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* 125, 2273–2282

26 Labosky, P.A. *et al.* (1994) Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* 120, 3197–3204

27 Tada, T. *et al.* (1998) Epigenotype switching of imprintable loci in embryonic germ cells. *Dev. Genes Evol.* 207, 551–561

28 Christofori, G. *et al.* (1995) Deregulation of both imprinted and expressed alleles of the insulin-like growth factor 2 gene during beta-cell tumorigenesis. *Nat. Genet.* 10, 196–201

29 McBride, G. (1997) Mom and pop genetics: genomic imprinting changes may illuminate cancer. *J. Natl. Cancer Inst.* 89, 1256–1258

30 Mori, M. *et al.* (1996) Relaxation of insulin-like growth factor 2 gene imprinting in esophageal cancer. *Int. J. Cancer* 68, 441–446

31 Nonomura, N. *et al.* (1997) Loss of imprinting of the insulin-like growth factor II gene in renal cell carcinoma. *Cancer Res.* 57, 2575–2577

32 Braude, P. *et al.* (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332, 459–461

33 Benirschke, K. and Kaufmann, P. (1990) *Pathology of the Human Placenta*, Springer-Verlag

34 Luckett, W.P. (1978) Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. *Am. J. Anat.* 152, 59–98

35 O'Rahilly, R. and Muller, F. (1987) *Developmental Stages in Human Embryos*, Carnegie Institution of Washington

36 Bain, G. *et al.* (1995) Embryonic stem cells express neuronal properties *in vitro*. *Dev. Biol.* 168, 342–357

37 Keller, G.M. (1995) *In vitro* differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* 7, 862–869

38 Klug, M.G. *et al.* (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J. Clin. Invest.* 98, 216–224

39 Brustle, O. *et al.* (1997) *In vitro* generated neural precursors participate in mammalian brain development. *Proc. Natl. Acad. Sci. U. S. A.* 94, 14809–14814

40 Burns, R.S. *et al.* (1983) A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc. Natl. Acad. Sci. U. S. A.* 80, 4546–4550

41 Jones, C.W. *et al.* (1980) Streptozotocin diabetes in the monkey: plasma levels of glucose, insulin, glucagon, and somatostatin, with corresponding morphometric analysis of islet endocrine cells. *Diabetes* 29, 536–546